

SOME MODEL STUDIES ON THE RETENTION AND REGENERATION OF NICOTINAMIDE COENZYMES

by

MATS-OLLE MÅNSSON



Lund 19800

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Abstract A4 A5 <u>Retention and regeneration of nicotinamide coenzymes</u> is a necessary requirement if dehydrogenases are to be used in <u>biotechnological systems</u>. Retention can be accomplished by <u>immobilization</u> and regeneration by enzymatic and non-enzymatic methods. NAD has been retained by co-immobilization with <u>liver alcohol dehydrogenase</u> and by coupling directly onto liver alcohol dehydrogenase. These preparations were active without addition of soluble NAD. Regeneration of the immobilized coenzyme was done with a <u>coupled substrate</u> system and a <u>coupled enzyme</u> system. <u>Immobilized acriflavin</u> has been used as a chemical reagent for regeneration of NAD in <u>enzyme reactors</u> with several dehydrogenases.			
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Date April 14, 1980

This thesis is based on the following publications, which will be referred to by their Roman numerals in the text.

- I. Continuous Regeneration of NAD(P)^+ by Flavins Covalently Bound to Sepharose.
Månsson, M.O., Mattiasson, B., Gestrelus, S. and Mosbach, K. (1976)
Biotech. Bioeng. 18, 1145-1159
- II. Preparation of an Alcohol Dehydrogenase-NAD(H)-Sepharose Complex Showing No Requirement of Soluble Coenzyme for its Activity.
Gestrelus, S., Månsson, M.O. and Mosbach, K. (1975)
Eur. J. Biochem. 57, 529-535
- III. Covalent Binding of an NAD-Analogue to Liver Alcohol Dehydrogenase Resulting in an Enzyme-Coenzyme Complex not Requiring Exogenous Coenzyme for Activity.
Månsson, M.O., Larsson, P.O. and Mosbach, K. (1978)
Eur. J. Biochem. 86, 455-463
- IV. Recycling by a Second Enzyme of NAD Covalently Bound to Alcohol Dehydrogenase.
Månsson, M.O., Larsson, P.O. and Mosbach, K. (1979)
FEBS Lett. 98, 309-313

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INTRODUCTION

In recent years enzymes have become increasingly important to the food and pharmaceutical industries (Brodelius 1978). The advantage of using enzymes as catalysts is that enzymes have high catalytic activity at neutral pH, moderate temperature and moderate pressure. For example, amylase and glucoamylase are used for converting starch to glucose and glucose isomerase is used in the production of high fructose syrup from glucose.

It is mainly hydrolytic enzymes of different kinds that have been used, partly because of the relative simplicity of their action, at least compared with that of the coenzyme-dependent enzymes.

Also in various analytic devices, such as enzyme electrodes (Guilbault 1976) and enzyme thermistors (Danielsson et al. 1979), enzymes have been used with success.

Of the 2122 enzymes given a specific number (Enzyme Nomenclature 1978), about one fifth are dehydrogenases requiring one of the coenzymes, NAD or NADP. This means that the NAD- and the NADP-dependent enzymes are potentially valuable in the future use of enzymes in synthesis, analytical devices and enzyme deficiency therapy (Lowe 1978, Mosbach 1978, Schmid 1979).

Better knowledge of these coenzymes and their interactions with dehydrogenases is therefore indispensable if full advantage is to be taken of dehydrogenases, and in a proposed EEC five year (1981-1985) programme for research and development in biomolecular engineering, coenzyme regenerating systems are recognized as an important research area (Walgate 1980).

Any enzymatic reaction catalyzed by a dehydrogenase requires the presence of a coenzyme to act as an electron carrier. This coenzyme is used in a stoichiometric amount relative to the substrate.

A dehydrogenase reaction: $S_1 + C_1 \rightleftharpoons S_2 + C_2$
 S_1 : oxidized substrate
 S_2 : reduced substrate
 C_1 : reduced coenzyme
 C_2 : oxidized coenzyme

There are two dissociable redox coenzymes used by dehydrogenases, viz the nicotinamide coenzymes, NAD and NADP. The chemistry of NAD and that of NADP are very similar, and what is said about NAD can often be applied also to NADP.

Because of the high cost of dehydrogenases and the necessity of using them in stoichiometric amounts, the use of NAD(P) dependent dehydrogenases in enzyme reactors requires the possibility of reusing the coenzymes (Baricos et.al. 1975).

The reuse of the coenzymes requires two things. First, retention, i.e. making it possible to separate and isolate the coenzyme from the substrate and product, and second, regeneration, i.e. oxidation or reduction of the redox coenzyme back to its original state after the reaction has taken place.

This thesis concerns the retention and the regeneration of the redox coenzyme couple NAD/NADH.

RETENTION OF NICOTINAMIDE COENZYMES

The molecular weight of NAD is 663, which means that the molecule is too small to be kept within a semipermeable membrane without hindering the substrate. The most common method to obviate this has been to increase the molecular weight by attaching the NAD molecule to a macromolecular support.

Many different polymers have been used as such supports for immobilization, e.g. the water-insoluble porous glass (Weibel et.al. 1971) and agarose beads like Sepharose (II, Larsson and Mosbach 1971, Zapelli et.al. 1976, Schmidt and Grenner 1976, Gacea and Whish 1978). Also water-soluble polymers have been used: dextrane (Larsson and Mosbach 1974, Schmidt and Grenner 1976, Gacea and Whish 1978), polyethyleneimine (Wykes et.al. 1975, Zapelli et.al. 1976), alginic acid (Coughlin et.al. 1976), polyacrylamide (Muramatsu et.al. 1977, Fuller et.al. 1980) and polylysine (Yamazaki et.al. 1976). The coenzyme has also been immobilized by coupling the NAD into a protein membrane together with an enzyme (Legoy et.al. 1978), and by coupling the coenzyme directly onto the enzyme together with which it works (III, IV, Venn et.al. 1977, Gacea and Venn 1979).

Modification of NAD

The immobilization of NAD usually requires modification of the coenzyme molecule before coupling, in order to obtain a coenzyme with a functional group, that can react with the polymer, distanced from the coenzyme with a spacer. Most modifications used, involve the nucleophilic attack at nitrogen N1 of the adenine ring followed by Dimroth rearrangement at high pH. This procedure yields a NAD-analogue with a substitution at the exocyclic N⁶ nitrogen of the adenine ring. This position is known to be less important in the binding to many dehydrogenases (Brändén et.al. 1975). The NAD-analogue, N⁶-[6-(aminohexyl)carbamoylmethyl]-NAD was the first functioning NAD-analogue synthesised according to the route illustrated in figure 1 (Lindberg et.al. 1973), and it has been used in the work described in papers II, III and IV.

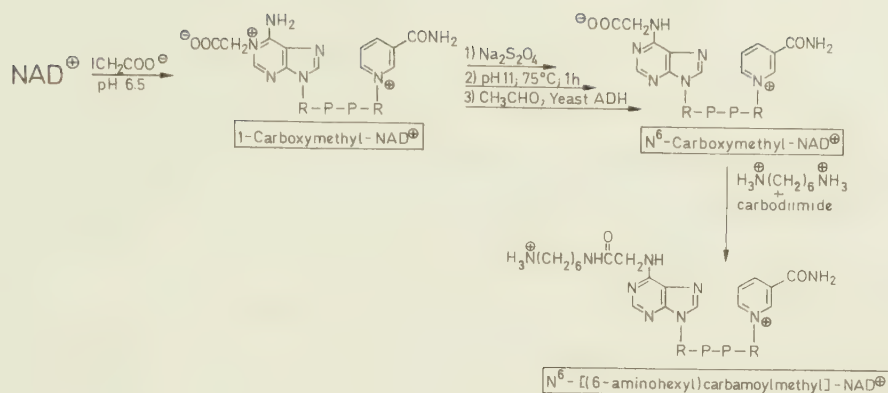


Figure 1. Synthesis of the NAD-analogue used in II,III and IV.

Also substitution at position C8 has been reported to give active coenzymes(Lee and Kaplan 1975,Zapelli et.al. 1976).

When NAD is substituted to be used in affinity chromatography it is only necessary for the coenzyme to be capable of binding to the enzyme; consequently the NAD can be substituted at several positions.

If the NAD is to be coenzymatically active,however,only substitution in the adenine ring is possible.Substitution at different positions in the adenine ring will also yield coenzyme analogues with different activity. E.g.,N1-carboxymethyl-NAD can be reduced with only a few per cent rate and N^6 -carboxymethyl-NAD with 60% rate, compared with native NAD (Lindberg et.al. 1973).

Immobilization of NAD

When the NAD thus has been modified it can be coupled to a polymer. The polymer has usually been activated to contain a reactive group, with which a functional group of the modified NAD can react.The most common activation methods are the cyanogen bromide method(Axén et. al. 1967) and the carbodiimide method (Larsson and Mosbach 1971). The cyanogen bromide activation involves the formation of a cyclic imidocarbonate on a carbohydrate polymer,to which an amino group can couple.In the carbodiimide method a carboxyl group yields an active ester,which reacts with an amino group to give a peptide bond.The immobilization of an NAD-analogue

Table 1. Relative reaction rates of different NAD-derivatives with YADH (yeast alcohol dehydrogenase), LADH (liver alcohol dehydrogenase), LDH (muscle lactate dehydrogenase) and MDH (heart malate dehydrogenase).The reaction rate for unmodified NAD is 1.0

Derivative	Reaction rate				Reference
	YADH	LADH	LDH	MDH	
N ⁶ -substituted NAD	0.61	-	0.76	-	Larsson and Mosbach 1974
" coupled to dextrane	0.16	-	0.14	-	"
" coupled to agarose	0.007	-	0.001	-	"
C8-substituted NAD	0.41	-	0.58	-	Zapelli et.al. 1976
" coupled to polyethyleneimine	0.47	-	0.03	-	"
" coupled to polylysine	0.05	-	0.06	-	"
N ⁶ -substituted NAD	0.23	0.58	0.25	0.58	Schmidt and Grenner 1976
" coupled to dextrane	0.085	0.175	0.24	0.185	"
" coupled to agarose	-	0.03	0.005	-	"
N ⁶ -substituted NADH coupled to polyacrylamide	0.37	0.28	0.045	0.26	Fuller et.al. 1980

which contains a vinyl group, is done in a polymerization reaction, where the analogue is mixed with other monomers.

Properties of modified and immobilized NAD

As mentioned earlier, the modification of NAD usually gives a coenzyme with a diminished activity with most dehydrogenases. The lower activity depends on the induced changes in coenzyme structure. It is thus the ability to bind to the enzyme that is influenced. Table 1 gives the relative reaction rates for different NAD derivatives.

Immobilization of the coenzyme has also other effects. The coupling of the coenzyme to a polymer will markedly retard diffusion rate of the coenzyme. A water-insoluble polymer will lower diffusion more than a water-soluble one.

It is not only diffusion that will suppress the coenzymatic activity, but also the fact that all immobilized coenzyme molecules are not equally accessible to the enzymes. Of the total amount of coenzyme immobilized, 80% of the dextrane bound (water-soluble) and 30% of agarose bound (water-insoluble) was reducible with yeast alcohol dehydrogenase (Larsson and Mosbach 1974).

Reducible coenzyme molecules may differ from each other also in rate of reduction. Owing to the steric influence of the polymer backbone some coenzyme can be reduced fast and others, slow. An enzyme can differ in affinity for the different coenzyme molecules of the same immobilized coenzyme preparation, i.e. the k_M can vary over a wide range.

The limitations discussed above also differ between different enzymes (table 1). Different dehydrogenases have different demands on the structure of the coenzyme.

REGENERATION OF NAD/NADH

One of the most important aspects of coenzyme regeneration is the specificity in the regeneration step, i.e. that the regeneration results in the desired compound (Schmid 1979). Too low specificity will soon give a mixture of active and inactive coenzymes (table 2). The inactive coenzymes may function also as highly potent inhibitors, since they may still have their binding ability.

Table 2. Number of cycles resulting in inactivation of 50% of the coenzyme at a certain regeneration specificity.

<u>Regeneration specificity</u>	<u>Coenzyme cycles</u>
90 %	7
99 %	69
99.9 %	690
99.99 %	6900
99.999 %	69000

It is clear from table 2 that the regeneration specificity must be 99.99% if half of the coenzyme is to be active after 6,900 cycles. The number of cycles required will depend on the nature of the alternative method for obtaining the same result. If the existing method is very good, the number of cycles required will be high, whereas a limited method can be replaced with a smaller number of coenzyme cycles.

The coenzyme NAD(H) can also be inactivated by the pH of the reaction solution. NAD and NADH are stable at different pH: NAD is stable at low pH and NADH at high (Lowry and Passoneau 1972). In enzymatic regeneration this is probably the major cause of coenzyme inactivation.

The different regeneration methods fall into the following groups.

1. Non-enzymatic regeneration

1.1. Chemical methods

1.2. Electrochemical methods

2. Enzymatic regeneration

2.1. Coupled enzyme methods

2.2. Coupled substrate methods

Non-enzymatic regeneration

Several known chemical reagents are capable of oxidizing NADH to NAD. They include derivatives of dihydropyridine, phenazine salts, 2,6-dichlorophenolindophenol and flavin derivatives (I, Jones and Taylor 1973, Chambers et.al. 1974, Venn et.al. 1977). Only sodium dithionite has been reported as a reagent for the regeneration of NADH from NAD in an enzymatic system (Jones et.al. 1972).

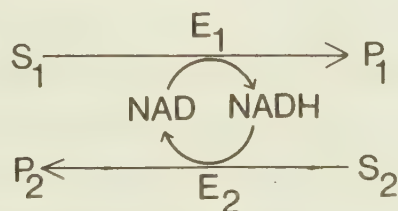
The main problem of non-enzymatic regeneration is the lack in specificity which soon inactivates many of the coenzyme molecules. The separation of the regeneration chemicals from the reaction solution may also be a problem, which can be overcome by polymerization (Chambers et.al. 1974) or by immobilization of the regeneration reagent (I).

Electrochemical regeneration of both NAD and NADH has been described (Aizawa et.al. 1975, Aizawa et.al. 1976). The advantage of the electrochemical regeneration is that it does not require addition of any reagents, only the electrode surface is required. The problems are lack of specificity, particularly in the reduction of NAD, and sometimes the enzyme of the system is impaired by the electrode reaction.

Enzymatic regeneration

There are two different enzymatic regeneration methods, coupled enzyme and coupled substrate regeneration.

Coupled enzyme regeneration:

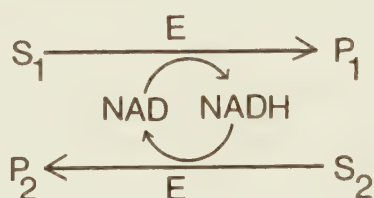


Scheme 1

As the name, coupled enzyme regeneration, implies, one enzyme, E_1 , reduces the coenzyme and another enzyme, E_2 , oxidizes the coenzyme back to its original state. At the same time two substrates, S_1 and S_2 , are converted to their respective products P_1 and P_2 .

There has been several reports on the use of coupled enzyme regeneration in model dehydrogenase reactor systems. In these reports different combinations of free/immobilized enzymes and free/immobilized coenzymes have been used (see table 3).

Coupled substrate regeneration:



Scheme 2

The coupled substrate regeneration method differs from the coupled enzyme method in that both the oxidation and the reduction of the coenzyme are done by the same enzyme. This requires that the enzyme has the ability to use alternative substrates. So far only liver alcohol dehydrogenase is known to have efficient substrate couples. Liver alcohol dehydrogenase, however, has a very broad substrate specificity and a variety of alcohols, ketones and aldehydes can serve as substrates (Sund and Theorell 1963), and this makes this enzyme very useful. Liver alcohol dehydrogenase has often been used in the stereospecific synthesis of compounds otherwise hard to obtain (Jones 1976, Davies and Jones 1979).

If the desired product of a coupled substrate system (scheme 2) is P_2 , then the co-substrate S_1 (an alcohol) must be used in excess if a good yield is to be obtained. The by-product P_1 (an aldehyde) is acetaldehyde if S_1 is ethanol. The reaction then can be promoted by driving the volatile acetaldehyde away from the system by bubbling nitrogen through it. In favourable cases both products P_1 and P_2 can be desired products.

Comparisons of different regeneration systems

Table 3 gives the results of different regeneration systems. It is a little difficult to compare the different systems since different amounts of enzyme has been used. A low coenzyme concentration will, of course, give a high coenzyme cycling rate more easily than a high coenzyme concentration.

Of the different chemical reagents used, all seem to give a similar cycling rate. The systems where polymerized FMN (Chambers et.al. 1974) or immobilized acriflavin (I) are used as regeneration reagents, have coenzyme cycling rates of the same magnitude as the free reagents.

In those cases where the same author investigated different combinations of free/immobilized coenzymes it can be seen that the free coenzyme functioned best and the immobilized coenzyme had a lower cycling rate. Of the immobilized coenzyme, the coenzyme coupled to a water-insoluble polymer like agarose is not as efficient as the one coupled to a water-soluble polymer.

The ratio of the cycling rates for free/water-soluble/water-insoluble coenzymes are 1/0.2/0.005 (Larsson and Mosbach 1974) or 1/0.1/0.03 (Wykes et.al. 1975). These differences in cycling rate depend largely on the much slower diffusion rates when the coenzyme is immobilized. Particularly when a water-insoluble polymer like agarose is used, the coenzyme can form a binary complex with the enzyme, which in the absence of competing free coenzymes can have a considerable life-time (Larsson and Mosbach 1974). The situation can be compared with that on an affinity chromatography column. The lower reaction rate can also depend on the fact that the accessibility of the coenzyme is diminished when the coenzyme is immobilized. The polymer to which the coenzyme is coupled imposes such restrictions that the coenzyme cannot interact in the desired way.

In systems with the coenzyme coupled to the enzyme (III, IV) the problem with coenzyme diffusion is obviated when a coupled substrate regeneration is used, since the coenzyme is permanently fixed at the active site. If the enzyme-bound coenzyme is regenerated with a second enzyme, the coenzyme will have to swing out of the active site of the liver alcohol dehydrogenase and into the active site of the second enzyme.

Table 3. Regeneration of NAD/NADH in enzymatic systems. The coenzyme is in its free form unless stated otherwise. The data in the table has in some cases been taken directly from the references and in other been calculated from results in the references.

<u>Regeneration system</u>	<u>Coenzyme cycling rate (h^{-1})</u>	<u>Coenzyme conc (mM)</u>	<u>Reference</u>
a. <u>Coupled enzyme regeneration</u>			
(YADH+LDH)	41	0.1	Larsson and Mosbach 1974
(YADH+LDH), immobilized NAD (dextrane)	7.4	0.1	"
(YADH+LDH), immobilized NAD (agarose)	0.2	0.1	"
(galDH+alDH), immobilized NAD (dextrane)	14	0.05	Davies and Mosbach 1974
(alDH+LDH), immobilized NAD (dextrane)	33	0.05	"
(YADH+LDH)	36	0.01	Wykes et.al. 1975
(YADH+LDH), immobilized NAD (PEI)	3.5	0.1	"
(YADH+LDH), immobilized NAD (agarose)	1.2	0.1	"
Immobilized (YADH+LDH)	12	0.01	"
Immobilized (YADH+LDH), immobilized NAD (PEI)	2.5	0.1	"
Immobilized (YADH+diaphorase)	4	0.5	Kawai et.al. 1975
(YADH+LDH, immobilized NAD (polylysine)	25	0.1	Yamazaki et.al. 1975
(YADH+diaphorase), enzyme and coenzyme in liquid membrane	0.5	0.65	May and Landgraft 1976
(YADH+MDH), immobilized NAD (PEI), microencapsulation	0.04	2.2	Campbell and Chang 1976
Immobilized (YADH+sorbitolDH)	1700	0.01	Chambers et.al. 1978
Immobilized (YADH+LDH)	29	$3 \cdot 10^{-3}$	Morikawa et.al. 1978
Immobilized (YADH+LDH), immobilized NAD (dextrane)	36	$3 \cdot 10^{-3}$	"
(LADH+LDH), NAD coupled to LADH	300	$1.2 \cdot 10^{-3}$	IV

Table 3 continued

<u>Regeneration system</u>	<u>Coenzyme cycling rate (h^{-1})</u>	<u>Coenzyme conc (mM)</u>	<u>Reference</u>
<u>b. Coupled substrate regeneration</u>			
LADH in hollow fiber reactor	160	1.0	Fink and Rodwell 1975
LADH and NAD co-immobilized	3400	$2 \cdot 10^{-4}$	II
NAD coupled to LADH	40000	$6 \cdot 10^{-6}$	III
LADH, immobilized NAD (polymerized)	1100	$2 \cdot 10^{-6}$	Fuller et.al. 1980
<u>c. Chemical regeneration</u>			
LADH+dihydropyridinium salt	1.5	0.1	Jones and Taylor 1973
LADH+2,6-dichlorophenolindophenol	0.2	1.0	"
LADH+flavin mononucleotide	2.0	0.1	"
Immobilized YADH+polymerized flavin mononucleotide	1.2	0.33	Chambers et.al. 1974
LDH+immobilized acriflavin	1.0	0.05	I
LADH+flavin mononucleotide	1.5	0.1	Jones and Taylor 1976

Abbreviations. YADH: yeast alcohol dehydrogenase, LADH: liver alcohol dehydrogenase, LDH: lactate dehydrogenase,
MDH: malate dehydrogenase, galDH: galactose dehydrogenase, alaDH: alanine dehydrogenase, sorbitolDH:
sorbitol dehydrogenase, PEI: polyethyleneimine

Another reason why it is difficult to compare different regeneration systems is that different amounts of enzyme have been used. If more enzyme is added, the system will usually function better. In those systems where the coenzyme is coupled to the enzyme (III, IV), the concentration of enzyme will vary with the concentration of coenzyme. If the substrate concentrations are constant, the same amount of product will be formed irrespectively whether the enzyme-coenzyme complex is in a large volume or in a small volume. This contrasts with systems with separated components where less product is formed on dilution. This fact was used to show that the activity before addition of soluble NAD to an enzyme-coenzyme complex really emanates from interactions between an enzyme molecule and a coenzyme molecule coupled to the very same enzyme (III).

There are also reports of NAD(H) regeneration where it is difficult to calculate the NAD concentration and the cycling number. A NAD-analogue has thus been coupled to lactate dehydrogenase (Venn et.al. 1977, Gacea and Venn 1979) with the bifunctional reagent glutaraldehyde. The stability of the complex was increased by reduction with sodium borohydride. The coenzyme was regenerated with the chemical reagents phenazineethosulphate and 2,6-dichlorophenolindophenol. The endogenous activity, i.e. activity before addition of soluble NAD was 0.8% of that after NAD addition. The enzyme-coenzyme complex contained about 6 NAD molecules per subunit of lactate dehydrogenase.

It has also been reported that NAD can be immobilized in a protein membrane together with alcohol dehydrogenase, to produce a system that shows enzymatic activity without addition of NAD (Legoy et.al. 1978).

There are only a few reports about electrochemical regeneration of NAD where an enzymatic reaction is incorporated in the system (Coughlin et.al. 1975). Such systems often have the disadvantage that the enzymatic reaction and the electrochemical reaction takes place in different compartments. This means that the cycling rate of the coenzyme is substantially lowered.

An analytic system where NAD has been immobilized on an electrode surface has recently been described (Yao and Musha 1979). This immobilized NAD could interact with lactate dehydrogenase and alcohol dehydrogenase. Addition of lactate and ethanol, respectively, produced immobilized NADH, which could subsequently be electrochemically oxidized to NAD.

IMMOBILIZED ACRIFLAVIN AS A NAD-REGENERATING SYSTEM (I)

Regeneration of NAD with coupled enzyme systems or with chemical reagents means addition of soluble components. This could be unnecessary if the regeneration system is immobilized. In an attempt to do this, acriflavin, not a flavin but a substituted acridine, was immobilized to epoxy substituted Sepharose.

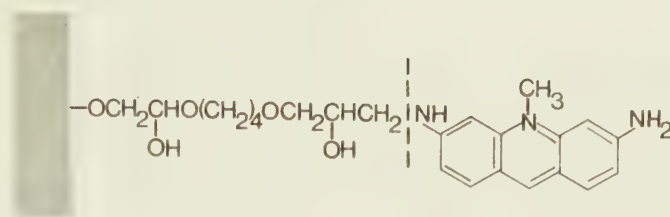
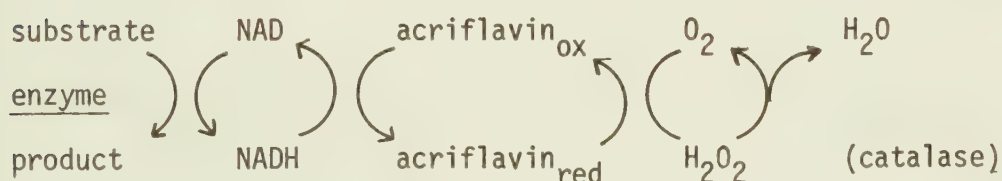


Figure 2. Acriflavin immobilized to epoxy substituted Sepharose.

Illumination on this preparation of immobilized acriflavin oxidized NADH to NAD. The reduced acriflavin was itself oxidized by oxygen and was thereby needed in only a catalytic amount. The immobilized acriflavin oxidized NADH with 25% efficiency compared with that of free acriflavin. Several dehydrogenases were tested in enzyme reactors together with immobilized acriflavin according to scheme 3.



Scheme 3

As can be seen in table 4, the immobilized acriflavin could oxidize the reduced coenzyme in reactors with both NAD and NADP dependent enzymes. In one experiment an enzyme reactor with lactate dehydrogenase was run for 80 hours. The coenzyme was then recycled 40 times and 225 times as much product was formed, as in a system without acriflavin.

Table 4. Immobilized acriflavin in enzyme reactors with different dehydrogenases. The increase in product formation is the ratio of the amount product formed in 2h in the presence and absence of acriflavin.

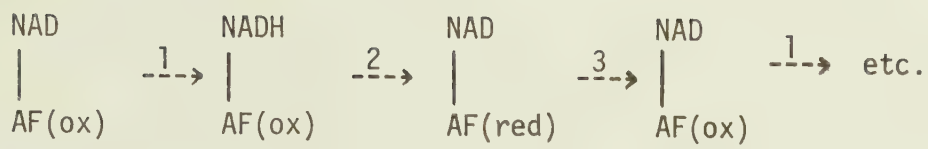
<u>Enzyme</u>	<u>Increase in product formation</u>
Lactate dehydrogenase	9.6
Alcohol dehydrogenase	5.6
Malate dehydrogenase	4.6
Alanine dehydrogenase	7.5
Glutamate dehydrogenase (NAD)	1.8
Glutamate dehydrogenase (NADP)	1.3

When the reduced flavins are oxidized by oxygen, hydrogen peroxide is formed (Holmström 1964). This hydrogen peroxide can be deleterious to the dehydrogenase, but the hydrogen peroxide can be converted to oxygen and water by addition of catalase or manganese oxide.

Also superoxide may be formed upon oxidation of reduced flavins (Massey et.al. 1971), and then superoxide dismutase can be added to remove the undesired substance.

An improvement in the NAD regeneration with acriflavin could be to make a bifunctional coenzyme, with one acriflavin part and one NAD part, which could serve as a coenzyme with a "built in" regenerating system (scheme 4). A prerequisite is, of course, that this new coenzyme is compatible with the enzyme. The advantage of this approach would be that the coenzyme would no longer have to diffuse to the regenerating system, and if one of the components were retained, the other would also be retained (Månsson 1977).

Bis-coenzymes with one nicotinamide part and one flavin part have been synthesised and used for electron transfer studies (Blankenhorn 1975).



1:enzyme+substrate

AF:acriflavin

2:light

3:oxygen

Scheme 4

CO-IMMOBILIZATION OF ENZYME AND COENZYME(II)

When NAD is immobilized to a solid support, the enzymatic regeneration is slow, particularly if both the enzyme and the coenzyme are immobilized. This would be overcome if the enzyme and coenzyme were co-immobilized in such a way that the coenzyme and the enzyme could interact. This is in analogy with earlier experiments where AMP and phosphorylase b were co-immobilized to give a permanently activated enzyme (Mosbach and Gestrelus 1974).

The co-immobilization was achieved by addition of a preformed binary complex of liver alcohol dehydrogenase and the NADH-analogue N^6 -[6-(aminohexyl)carbamoylmethyl]-NADH, to a cyanogen bromide activated agarose matrix. The reduced coenzyme was used because of its lower dissociation constant ($0.5 \mu M$) compared with that of the oxidized coenzyme, thus assuring that the coenzyme is in the active site the major part of the time.

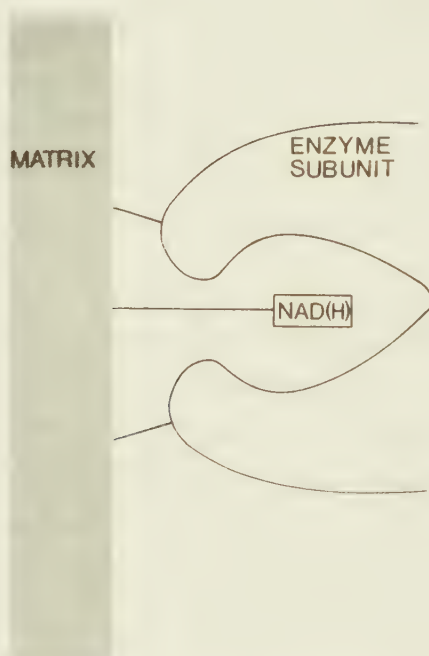


Figure 3. Schematic drawings of co-immobilized NAD(H) and liver alcohol dehydrogenase

The resulting enzyme-coenzyme-solid phase complex (figure 3) was enzymatically active without addition of soluble coenzyme. The activity was up to 40% of that obtained when an excess of free coenzyme was added. The enzymatic activity was measured with a coupled substrate assay which at the same time regenerated the coenzyme with a maximum cycling rate of 3400 cycles per hour. Coupled enzyme regeneration or chemical regeneration was impossible or very much less effective. This is probably due to the steric influence of the matrix, shielding off the active site and the coenzyme from the bulk solution.

This kind of co-immobilized enzyme and coenzyme is simple to prepare and should be useful in e.g. the synthesis of stereospecifically labelled radioactive compounds (Battersby et.al. 1975).

The thermostability of liver alcohol dehydrogenase was increased when co-immobilized with NADH. After one hour at 50°C, the activity of the co-immobilized enzyme had decreased to 70%, whereas the activity of liver alcohol dehydrogenase not co-immobilized fell to 20% of its original value. The activity of the enzyme in solution was completely lost after one hour at 50°C.

This method of co-immobilization is probably useful per se also for other enzymes, but since only coupled substrate regeneration is possible, it is restricted to liver alcohol dehydrogenase, which is the only enzyme for which efficient substrate couples are hitherto known.

COENZYME COUPLED TO ENZYME (III,IV)

The system with co-immobilized enzyme and coenzyme had the disadvantage that coupled substrate regeneration was the only possible regeneration method. An alternative way would then be to couple the coenzyme directly to the enzyme. This would eliminate the steric influence of the matrix.

In nature there are such enzymes which have a covalently bound coenzyme as a prosthetic group. Those systems include succinate dehydrogenase with a covalently bound 8 α -histidyl-FAD (Walker et.al. 1972) and citrate(pro-3S)lyase from Klebsiella Aerogenes with a covalently bound coenzyme A (Robinson et.al. 1976).

A soluble enzyme-coenzyme complex was achieved by coupling the NAD-analogue N⁶-[6-(aminohexyl)carbamoylmethyl]-NAD, containing a terminal amino group, to a carboxyl group at liver alcohol dehydrogenase, in a carbodiimide mediated reaction. This enzyme-coenzyme complex consisted of about one NAD molecule per enzyme subunit, and it was enzymatically active without addition of free NAD. The activity without addition of free NAD, measured in a coupled substrate assay, was 25% of that obtained in the presence of free NAD in excess. The coupled substrate regeneration of the enzyme-bound coenzyme was the most efficient, but the coenzyme was also accessible to both other enzymes and chemical reagents. The enzymatic regeneration was much slower than the coupled substrate regeneration (300 h⁻¹ and 40000 h⁻¹ respectively), but the possibility of coupled enzyme regeneration makes the use of the enzyme-coenzyme complex more versatile.

The NAD(H)-liver alcohol dehydrogenase complex can regenerate both the oxidized and the reduced coenzyme, which can subsequently be used by the second enzyme.

In the enzyme-coenzyme complex of liver alcohol dehydrogenase and NAD each subunit contains, on the average, one NAD molecule. Not all NAD molecules are active and it is therefore assumed that there is at least one carboxyl group at the enzyme that leads to an inactive coenzyme.

The amino acids that are possible points of attachment for the active NAD-analogue are asp 223, asp 273, asp 277 and glu 366. Asp 273 would give an enzyme-coenzyme complex where the interactions between the active

site and coenzyme were the same as in a normal binary complex. Coupling to the other amino acid residues, however, would require that the normal binary complex was somewhat distorted with respect to binding of the adenine part of the coenzyme (C.I. Brändén, personal communication).

A way to direct the coupling to the carboxyl group, which leads to an active coenzyme, would then be to make sure that the coenzyme is situated in the active site when the coupling takes place, i.e. in accordance with the principles of affinity labelling.

This was accomplished by forming a ternary complex between the enzyme, the NAD-analogue and pyrazole (Theorell and Yonetani 1963). Isobutyramide and ethanol could be substituted for the pyrazole since ethanol converts the NAD to NADH. Liver alcohol dehydrogenase, NADH and isobutyramide would then form a strong ternary complex (Andersson et al. 1978).

The coupling agents were added when all the sites were occupied by an NAD-analogue. Experiments carried out in this way, however, did not result in any coupling at all.

A possible interpretation of this result could be that coupling takes place only when the coenzyme to be coupled is not in the active site. This view is supported by the results of another experiment, where equimolar concentrations of NAD-analogue and unmodified NADH were present during coupling. The much lower dissociation constant of NADH (about 150 times) ensures that all the active sites are occupied with an unreactive NADH molecule. The result of such a coupling was similar to that obtained without the presence of NADH.

Another interpretation of the failure to get any coupling when the NAD-analogue was in the active site as a ternary complex is that the ternary complex forming molecule sterically hinders the coupling reaction. This interpretation is favoured by the result of a coupling reaction where NAD-analogue was used in excess at the same time as 0.1 M pyrazole was present. This experiment gave a very low coupling result indicating that the pyrazole was involved.

As mentioned earlier, asp 223 is a possible point of attachment for an active NAD-analogue. This amino acid residue normally also forms a hydrogen bond with the adenine ribose of the coenzyme (Brändén et al.

1975). This means that when NAD is in the active site, the asp 223 is blocked and no coupling can take place. The coupling can occur only when the active site is unoccupied and when pyrazole or another ternary complex forming molecule is present, the coenzyme is bound very firmly and the active site is always occupied, thus making it impossible for the coupling reaction to take place. The binary complexes between liver alcohol dehydrogenase and NAD or NADH are not so strong as the ternary complex and in these cases the active site is sometimes vacant and the coupling can occur.

This idea is compatible with the supporting results of the two other interpretations and is also combined with an amino acid residue known to be a possible point of attachment.

If the active NAD-analogue is covalently bound to asp 223, the coenzyme will no longer have any hydrogen bond to asp 223. Therefore the normal binding has been distorted and the binding role of the adenosin part of the coenzyme has partly been replaced by the covalent bond between the enzyme and the coenzyme.

Affinity experiments with the enzyme-coenzyme complex

When the affinity labelling approach to get a pure enzyme-coenzyme complex, i.e. a complex with one correctly coupled coenzyme per enzyme subunit, failed, affinity chromatography was tried as a means of purifying the existing complex.

This was done in two affinity chromatography experiments with different ligands. First, an affinity column with immobilized NADH was used (Andersson and Mosbach 1979). The retardation on the column would be dependent on how many active sites (one or two) of the alcohol dehydrogenase (a dimer) were occupied with a correctly coupled NAD. With both active sites blocked by a NAD, the enzyme would be unretarded, whereas the enzyme with no NAD at all would be more retarded.

The experiment resulted in a certain fractionation of the sample. The endogenous activity of the first fractions was a little higher than that of the sample applied. The last fractions had no endogenous activity at all. No absolute separation occurred.

In another affinity experiment a column with an immobilized C_{11} fatty acid was used. The idea was that the fatty acid was to function as a ternary complex forming molecule in analogy with experiments with immobilized pyrazole (Lang and Vallee 1976). The formation of ternary complex between fatty acid, NAD and enzyme on the column would retard those enzyme molecules with one or two subunits with NAD in the active site.

It could be suspected that the fatty acid-column would function as a hydrophobic chromatography column and that effect also was found to be the predominating one, and the first protein to be eluted had 100% endogenous activity, but only a few per cent of the added protein were in those fractions.

It is probably difficult to obtain any substantial amounts of pure enzyme-coenzyme complex in this way. This is probably mainly because the liver alcohol dehydrogenase is a dimeric enzyme, and enzyme molecules where both subunits are substituted with a properly attached coenzyme are scarce. Particularly since it is assumed that only every fourth active site has a correctly attached NAD in the active site.

Even if the results of these affinity experiments were poor with respect to purification, they were important because they showed that the normal coupling procedure gives both enzyme molecules with 100% endogenous activity, and enzyme molecules with no endogenous activity at all.

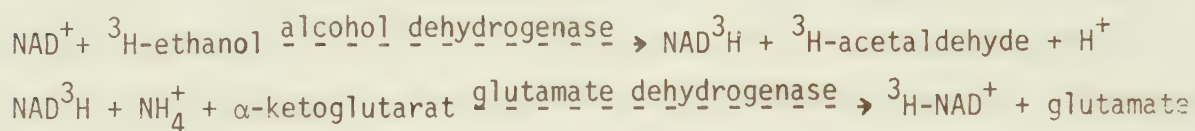
A technique for rapid analysis of enzyme-coenzyme complex

The usual way to analyze the results of a coupling experiment has hitherto been to dialyze the reaction sample and then to separate uncoupled coenzyme from the enzyme-coenzyme complex by gel filtration. This is not a very suitable system if different coupling conditions are to be screened. Every experiment would take about three days and only one experiment could be carried out at a time. Therefore, a more rapid way for determining the coupling result was developed.

In that system a radioactively labelled coenzyme was used. After a completed reaction a sample with a known amount of protein was applied on a silica TLC-plate and the TLC was developed in isobutyric acid(5): NH_3 (3): EDTA(sat). The protein with coupled radioactive coenzyme was retarded on the application point and the coenzyme had a R_f value of 0.5. The

protein spot on the TLC plate was cut out, 1M NH_3 added, and finally the radioactivity was determined in a liquid scintillation counter. Since the specific radioactivity of the coenzyme was known, the coupling yield could be determined.

Tritium labelled coenzyme analogues were synthesized enzymatically using ^3H -labelled ethanol as radioactive starting material, according to this scheme (Larsson and Månsson 1978):



Since alcohol dehydrogenase is an A-side specific enzyme and glutamate dehydrogenase a B-side specific enzyme in the reduction/oxidation of the coenzyme (You et.al. 1978), the label of the final product is stable as long as it is used with A-side specific or B-side specific dehydrogenases only. If the coenzyme is used in a cycle with both A-side and B-side specific enzymes, the label is lost.

A typical labelling experiment was carried out as follows: The reaction mixture consisted of 5 mM NAD-analogue, 2.5 mM ethanol (100 Ci/mol), 50 mM $(\text{NH}_4^+)_2$ α -ketoglutarat, 50 mM sodium phosphate pH 7.5 and 1.5 U each of alcohol dehydrogenase (horse liver) and glutamate dehydrogenase (bovine liver). The reaction was followed on TLC and by radioactivity measurement of the coenzyme spot. After 3.5 hours the reaction was judged as complete to equilibrium. The reaction was then applied to an ion-exchange column (Dowex 50WX2) and the product was eluted with a gradient of NH_4HCO_3 , pH 7.5. The yield was 7.7 μmoles with $4 \cdot 10^7$ dpm/ μmole . The radioactivity can perhaps be increased by adding aldehyde dehydrogenase (A-side specific) to the reaction solution and then use all the tritium label, in analogy with a recent report (Viola et.al. 1979) where a combination of alcohol dehydrogenase and aldehyde dehydrogenase was used to prepare deuterated NADH.

Possible future development with enzyme-coenzyme complex

It has been shown that NAD covalently bound to liver alcohol dehydrogenase can be oxidized and reduced by other enzymes, e.g. lactate dehydrogenase.

A further development of this approach would be to link the lactate dehydrogenase to the enzyme-coenzyme complex. Ideally the enzymes should then be directed in such a way that the active sites are facing one another.

In a recent report (Bourdillon et.al. 1979), glucose oxidase has been covalently coupled to an electrode surface and the hydrogen peroxide formed in the enzymatic reaction could be measured amperometrically. A similar approach is possible with the enzyme-coenzyme complex, coupled to a glassy carbon electrode (Torstensson et al. 1980). Addition of ethanol to the solution produced NADH, which could be electrochemically oxidized to NAD. However, only one cycle was possible. The electrochemical oxidation of the enzyme-bound NADH was also possible when the complex was free in solution.

As mentioned earlier, the adenosine part of the NAD-analogue that is covalently bound to liver alcohol dehydrogenase is perhaps not positioned in the same way in the active site as a non-covalently bound coenzyme. If the binding role of the adenosine part of the coenzyme has been partly replaced with the covalent bond, the adenine part is no longer necessary. This means that it would perhaps be enough if a covalently bound coenzyme consisted of nicotinamide nucleotide, the negative charge of the phosphate bridge and a spacer arm with a reactive group for coupling to the enzyme. The nicotinamide ribose and the phosphate bridge are important parts in the orientation of the coenzyme in the active site (Brändén et.al. 1975). A necessary condition is that the spacer has the length that makes possible the proper interactions between the coenzyme-analogue and the active site.

CONCLUSIONS

In every individual case where a coenzyme regenerating/retaining system is considered, a special assessment must be made. This assessment must take into account all factors, such as how many coenzyme cycles are required, and what restrictions do the enzymes impose on the coenzyme.

The most immediate applications of regeneration/retention systems probably lie in the field of analytic systems. The use in the synthesis of stereospecific substances may also prove useful.

The use of an immobilized regeneration reagent may find its use where a limited number of coenzyme cycles is required, since the chemical regeneration is less specific in the regeneration step.

The more sophisticated systems with co-immobilized enzyme and coenzyme and coenzyme coupled to enzyme give a much higher specificity in the regeneration. The co-immobilized system has its limitations in the fact that only coupled substrate regeneration is possible, but in return it is simple to prepare and the co-immobilization stabilizes the enzyme against thermal inactivation.

Liver alcohol dehydrogenase is hitherto the only dehydrogenase for which efficient substrate couples are known. Future use of coupled substrate regeneration systems with other dehydrogenases requires a search for dehydrogenases with this property. Such dehydrogenases may perhaps be found among bacteria.

The enzyme-coenzyme complex between NAD and liver alcohol dehydrogenase also functions best with a coupled substrate regeneration, but coupled enzyme regeneration is also possible, which makes such a system more versatile than the co-immobilized one. The enzyme-coenzyme complex may be used also to get enzymological information about coenzyme-enzyme interactions and using different NAD-analogues. Information about enzyme surface and distances between active site and certain amino acid residues.

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